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Sertifikaat

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the documents attached hereto are true copies of the Forms P2, P6 provisional specification and drawings of South African Patent Application No.2002/9830 in the names of University of Cape Town; South African Medical Research Council

Filed : 04 December 2002

Entitled : A method for the production of HIV-1 Subtype C PR55 GAG Virus-like particles

Geteken te PRETORIA in die Republiek van Suid-Afrika, hierdie 12th dag van January 2004

Signed at PRETORIA in the Republic of South Africa, this 12th day of January 2004

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APPLICATION FOR A PATENT

AND ACKNOWLEDGEMENT OF RECEIPT
(Section 30 (1) – Regulation 22)

The granting of a patent is hereby requested by the undermentioned applicant on the basis of the present application filed in duplicate

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21	01	2002/9830	

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TITLE OF INVENTION

54	A METHOD FOR THE PRODUCTION OF HIV-1 SUBTYPE C PR55 GAG VIRUS-LIKE PARTICLES

THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2. THE EARLIEST PRIORITY CLAIM IS:

COUNTRY:	NUMBER:	DATE:
NIL	NIL	NIL

~~THIS APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO.~~

21	01	

~~THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND IS BASED ON APPLICATION NO.~~

21	01	

THIS APPLICATION IS ACCOMPANIED BY:

- ☒ 1. A single copy of a provisional specification of 25 pages.
- ☒ 2. Drawings of 4 sheets.
- ☐ 3. Publication particulars and abstract (Form P.8 in duplicate).
- ☐ 4. A copy of Figure of the drawings (if any) for the abstract.
- ☐ 5. Assignment of invention.
- ☐ 6. Certified priority document.
- ☐ 7. Translation of the priority document.
- ☐ 8. Assignment of priority rights.
- ☐ 9. A copy of the Form P.2 and the specification of S.A. Patent Application No .
- ☐ 10. Declaration and power of attorney on Form P.3.
- ☐ 11. Request for ante-dating on Form P.4.
- ☐ 12. Request for classification on Form P.9.
- ☒ 13. Form P.2 in duplicate.
- ☐ 14. Other.

74	ADDRESS FOR SERVICE: SPOOR & FISHER, SANDTON

Dated: 4 December 2002

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RECEIVED
REGISTRAR OF PATENTS, DESIGNS, TRADE MARKS AND COPYRIGHT
2002-12-04
REGISTRATEUR VAN PATENTE, MODELLE, MERKE SKRYWE EN OUTEURSREGTE
REGISTRAR OF PATENTS

FORM P.2

REPUBLIC OF SOUTH AFRICA	REGISTER OF PATENTS	PATENTS ACT, 1978

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TITLE OF INVENTION

54 A METHOD FOR THE PRODUCTION OF HIV-1 SUBTYPE C PR55 GAG VIRUS-LIKE PARTICLES

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ADDRESS FOR SERVICE	S & F REF
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SPOOR & FISHER

FORM P.6

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

PROVISIONAL SPECIFICATION

(Section 30(1) – Regulation 27)

OFFICIAL APPLICATION NO.		LODGING DATE	
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TITLE OF INVENTION

54	A METHOD FOR THE PRODUCTION OF HIV-1 SUBTYPE C PR55 GAG VIRUS-LIKE PARTICLES

A METHOD FOR THE PRODUCTION OF HIV-1 SUBTYPE C PR55 GAG VIRUS-LIKE PARTICLES

BACKGROUND OF THE INVENTION

THIS invention relates to a method for the production of HIV-1 subtype C Pr55 Gag virus-like particles, to the virus-like particles prepared by the method, and to the use of the virus-like particles in a vaccine.

The HIV genome contains three open reading frames. The *gag* open reading frame (Fig. 1) encodes a 55 kDa precursor protein ($Pr55^{Gag}$) which is cleaved further by an HIV-encoded protease during virion maturation into three major structural proteins, a regulatory domain and 2 spacer peptides (Luciw, 1996). The structural proteins include the matrix (MA) protein (P17 – AA1 to AA132), the capsid (CA) protein (P24 – AA133 to AA363) and the nucleocapsid (NC) protein (P9 – AA377 to AA432). The regulatory domain (P6) spans AA449 to AA500 while the spacer regions P1 and P2 are located from AA433 to AA448 and AA364 to AA376 respectively (von Schwedler *et al.*, 1998).

The *pol* open reading frame overlaps that of *gag* from AA430 and is expressed via a ribosomal frame-shifting event that occurs at a frequency

of 5 to 10% during translation to produce a Gag-Pol precursor protein of 160kDa (Pr160) (Jacks *et al.*, 1988). The *pol* gene encodes several open reading frames including that for the protease, reverse transcriptase, RNase H and integrase enzymes of HIV-1. The *env* open reading frame lies further downstream of *pol* and encodes a 160 kDa precursor protein (gp160) of the viral envelope proteins gp41 and gp120 (Luciw, 1996).

After infection of a host cell, HIV-1 RNA is reverse transcribed into DNA which is subsequently integrated into the host genome (proviral stage). The Gag and Gag-Pol precursors are translated from transcribed HIV-1 provirus RNA in the cytosol and targeted to the host cell membrane. The Gag precursor associates with two copies of viral RNA and interacts with the Gag-Pol precursor to assemble into particle-like structures which line the host-cell membrane. They aggregate in such a way as to induce membrane curvature and subsequent bud formation during which viral Env proteins are also incorporated into the forming particles. The particles pinch off the membrane after which the HIV-1 particle maturation occurs, with the protease cleaving Gag and Gag-Pol into mature structural and functional proteins which lead to core condensation and thus a mature infectious virion.

Gag has been shown to assemble into virus-like particles (VLPs) in the absence of any other HIV-encoded genes in both mammalian and insect cells. These particles closely resemble the morphology of immature HIV virions and are non-infectious (Overton *et al.*, 1989; Gheysen *et al.*, 1989; Royer *et al.*, 1991; Royer *et al.*, 1992; Shioda and Shibuta, 1990; Vernon *et al.*, 1991; Mergener *et al.*, 1992). A number of Gag domains have been shown to be important in driving this particle assembly process and it has been shown that in fact about 80% of this precursor protein can be either deleted or replaced by heterologous sequences without significantly compromising VLP production (Accola *et al*, 2000). These important domains are discussed below with respect to the functions of the individual proteins comprising Gag.

MA protein (p17)

The MA domain of Gag (Figure 2.) comprises a total of 132 amino acids and is responsible for targeting Gag precursor protein to the plasma membrane and virus-like particle assembly. The M domain (retrovirus membrane-binding domain) at the N-terminal of MA is mostly responsible for this function. MA has an N-terminal glycine residue which has been shown to be required for targeting Gag to the host cell membrane and facilitating particle assembly (Gheysen *et al.*, 1989). For this to occur, the glycine residue has to be myristylated. The amino acid recognition sequence for myristylation to occur at the N-terminus of Gag is gly-x-x-x-ser/thr.

The targeting and accumulation of HIV-1 Gag precursor at the host cell membrane by myristylation has been shown to occur in baculovirus-infected yeast cells, insect cells and mammalian cells (Jacobs *et al.*, 1989; Gheysen *et al*, 1989; Bryant and Ratner, 1990). Substitution of the glycine residue erradicated particle formation, complementation of the residue restored VLP production and when using myr⁻ mutants, Gag precursor was shown to accumulate in infected cell cytoplasm but did not associate with the host cell membrane. The myristyl moiety is thus required for stable membrane association of the particles. Only complete inhibition of Gag myristylation prevents VLP budding (Morikawa *et al.*, 1996), i.e. only a few myristylated Gag molecules are sufficient for plasma membrane targeting and budding.

Spearman (1997) has shown that myristate is the primary determinant of Gag-host cell membrane stability and therefore critical for particle assembly. Paillart and Göttlinger (1999) have proposed a model from results showing that the N-terminus of Gag is critical for insertion into the host cell membrane.

Apart from the myristylation signal other regions of MA have been shown to be important for targeting Gag to the host cell membrane and subsequent

particle assembly. Fäcke *et al.* (1993) showed that a large deletion of MA (AA16 to 99) caused drastic alteration of particle morphogenesis leading to immature particles produced in the endoplasmic reticulum instead. MA is required for the proper assembly of envelope proteins into the virion.

Yuan *et al.* (1993) showed that various MA deletions and substitutions caused a dramatic reduction in virus particle production. They demonstrated that it is possible that a polybasic region in MA (AA20 to 32) serves as part of a Gag transport signal to the membrane.

Zhou *et al.* (1994) studied this polybasic region further and showed that the highly basic residues form a positively charged surface which interacts with negatively charged phospholipids on the inner face of the lipid bilayer of the plasma. Ono and Freed (1999) have shown that a single mutation of MA (AA6 from V to R) severely impaired membrane binding without affecting myristylation.

Capsid (CA) protein (p24)

The CA domain (Figure 3.) encodes a protein of approximately 230 amino acids in length and has several domains which appear to be important for particle assembly, the first of which is a major homology region (MHR). The region extending from the N-terminus of CA downstream to the MHR is dispensible for particle formation, but any further deletions extending further into the MHR impair particle production (Borsetti *et al.*, 1998). Zhao *et al.*, (1994) also showed that baculovirus constructs of HIV-1 CA with a 10-amino acid deletion of AA140-150 as well as a separate deletion of AA250-260 led to the accumulation of viral protein at the cell membrane of insect cells. However there was no particle assembly or extracellular budding indicating that these two regions of CA at least, must play some role in normal particle formation.

On the other hand, Borsetti *et al.* (1998) showed that efficient particle formation occurred in the absence of both MA (excluding the myristyl anchor) and the N-terminal of the CA domain and therefore concluded that

there is no distinct region between the myristyl anchor and MHR which is absolutely essential for efficient particle release or assembly. They concluded that the C-terminal half of Gag contains protein-protein interaction domains which are essential for efficient particle assembly.

It seems that the C-terminal sequences may be required for protein-protein interactions but are not required for spherical particle formation and that the sphere is determined by the presence of an N-terminal extension on the CA domain.

The presence of RNA (heterogeneous in size and of viral and cellular origins) within the particles has been reported (Gheysen and Shioda and Shibuta)

Truncations into the P2 spacer regions have been shown to abolish particle formation.

Spacer region 2 (p2)

Borsetti *et al.*, 1998 have shown that the presence or absence of p2 determines the assembly of Gag proteins into spherical particles or cylindrical particles respectively. Morikawa *et al.* (2000) have also verified that this region is essential for VLP production in that if this region is truncated in any way, VLP production is abolished.

Nucleocapsid (NC) protein (p7)

The NC domain (Figure 4.) has been shown to contain two well-conserved Cys-His boxes resembling zinc finger motifs often found in DNA binding proteins. These are thought to play a role in RNA binding and encapsidation but influence some other aspects of particle assembly as well. There are two highly basic regions flanking these two motifs which have been shown to influence RNA binding *in vitro* and RNA encapsidation into virions if mutated. Jowett *et al.* (1992) showed that the deletion of the second Cys-His box did not affect particle formation but reduced RNA

binding substantially. However, they also showed that deletion of both Cys-His boxes encouraged the formation of larger particles and the loss of RNA binding altogether. The deletion of sequences upstream of the Cys-His boxes caused the abolition of particle-forming ability.

Dawson andYu (1998) showed that the NC domain is essential for efficient assembly of HIV-1 and for the production of particles with wildtype density.

In addition to the Cys-His boxes, an I domain (interaction or assembly domain) close to the N-terminal of NC has been identified which is responsible for the formation of Gag protein complexes and also for the formation of punctate foci of Gag proteins at the plasma membrane. There are two positively charged basic arginine residues (AA380 and AA384) which have been shown to be critical for the function of the N-terminal I domain (interaction or assembly domain). Sandefur *et al.* (2000) have shown that I domain-deficient mutants block the formation of budding VLPs.

Spacer region 1 (p1)

CA and NC are separated by a short spacer region SP1 which is a protease cleavage site. Wiegers *et al.* (1998) have shown that when cleavage of SP1 from NC is prevented, maturation of particles is delayed and the ribonucleoprotein core has an irregular morphology. However, when SP1 cleavage from CA is prevented, normal condensation of the ribonucleoprotein cone occurs but capsid condensation is prevented. They concluded from this that HIV maturation is a sequential process controlled by the rate of cleavage at individual sites.

P6 protein

Elements important for controlling particle size are contained within the C-terminal region of gag (P6) (Figure 5) as various deletions and substitutions of this region have been shown to induce the formation of very large particles (Garnier *et al.*, 1998). A specific domain referred to as the late (L) domain has been identified in P6 that is critical for the virus-cell separation

step. This region contains a PTAPP amino acid sequence. Sequences downstream of this domain in P6 were shown to be dispensable for virus release.

Other features of Gag influencing VLP formation

Buck *et al.* (2001) have recently found that mRNA of the HIV-1 Gag open reading frame exhibits internal ribosome entry site (IRES) activity that promotes translational initiation of Gag, producing a 40 kDa Gag protein. This IRES is located at an internal AUG codon found near the N-terminus of CA. This may have consequences when Gag is expressed in *in vitro* systems since initiation of translation is thought to be promoted by the direct binding of ribosomes with the participation of other host cell factors quite independently of the mRNA cap.

Minimal HIV-1 *gag* sequences required for VLP assembly and release

Although numerous domains of Gag have been shown to perform a particular function in Gag VLP assembly and release, deletion mutants of Gag have indicated that a number of these can be dispensable, and assembly of VLPs has been shown to be surprisingly tolerant to significant modifications of Gag protein (Wang *et al.*, 1998; Accola *et al.*, 2000; Wilk *et al.*, 2001). Wang *et al.*, (1998) made a series of C-terminal truncated mutants with which to examine VLP-producing capabilities. Truncated Gag precursors lacking most of the C-terminal Gag assembled into particles and were released from mammalian cells. A mutant with most of MA and the entire p6 region deleted still produced particles although less than wildtype particles. The smallest Gag product capable of VLP assembly was a 28 kDa protein which consisted of a few MA amino acids and the CA-p2 domain. The N-terminal portions of CA appeared to be critical when most of the MA domain was deleted, suggesting a requirement for an intact CA domain to assemble and release particles. Accola *et al.* (2000) showed that 80% of Gag could be deleted or replaced by heterologous sequences without significantly compromising VLP production. The smallest chimeric molecule still able to efficiently form VLPs was 16 kDa. This construct

contained a leucine zipper domain of the yeast transcription factor GCN4 to substitute for the assembly function of nucleocapsid, followed by a PPPPY motif to provide the L domain function, and retained only the myristylation signal and the C-terminal CA-p2 domain of Gag.

Ability of Gag to stimulate an immune response

The long-term solution to combating the AIDS epidemic is through immunisation with a suitable vaccine and there are numerous HIV-1 genes and epitopes which are currently being used to develop vaccines. Although there is no Gag vaccine in use to date, a number of studies have shown that this protein induces an immune response in HIV-1-infected patients as well as in animals challenged with novel vaccines (Friedman *et al.*, 2000; Revskaya and Frankel, 2001).;).This response has been shown in some cases to be both humoral and cell-mediated (Qui *et al.*, 1999; Leung *et al.*, 2000; Qui *et al.*, 2000; Montefiori *et al.*, 2001; Kazanji *et al.*, 2001).

Goulder *et al.* (2000) did studies to identify the epitopes, which dominate the CTL response in ethnic groups and age groups worst hit by the global epidemic. They focussed on Gag because a number of gag specific responses have been shown to be associated with protection in HIV infection (Nixon and McMichael, 1991; Riviere *et al.*, 1995). The immunodominant Gag-specific CTL responses appeared to be focussed on 3 highly immunogenic regions, which together spanned 16% of the total length of Gag p17 and Gag p24 proteins but which represent two thirds of the dominant Gag-specific CTL responses detected. These results suggest that Gag would be an ideal candidate for vaccine design.

Plants as sources of vaccines

There are many examples of the use of plants as sources of foreign protein and they are considered as viable and competitive expression systems for large-scale protein production (Doran, 2000). Favourable reasons for their use include the potential for large-scale, low-cost biomass production, a low risk of contamination by mammalian viruses and other animal pathogens,

the ability of plant cells to correctly fold and assemble multimeric proteins, and a low processing requirement for proteins administered orally in plant food or feed. They are thus considered a viable option for the production of foreign proteins, which can be used as vaccines.

A number of potentially useful vaccine candidates have been produced in plants and tested in animals. A useful technique for introducing foreign genes into plants has been via viral vector transmission.

Usha *et al.*, (1993) have used cowpea mosaic virus (CPMV) particles to express epitopes of foot and mouth disease virus (FMDV) on their coat protein as a result of a fusion on the coat protein gene.

Koo *et al.* (1999) made hybrids of tobacco mosaic virus (TMV) by fusing short epitopes from murine hepatitis virus (MHV) to TMV coat protein and subsequently propagating them in tobacco plants.

Fernández-Fernández *et al.* (2001) have developed a plum pox potyvirus vector for the expression of foreign proteins. They have used it to express an antigenic structural protein of rabbit hemorrhagic disease virus (RHDV), producing chimeric virus particles which when inoculated into rabbits produce an immune response against RHDV.

A number of other methods have been used to produce vaccine candidates in plants. McCormick *et al.* (1999) demonstrate the modification of a TMV vector such that it not only produces single chain Fv fragments in plants, but secretes them into the apoplast. This makes harvesting of the product a lot simpler than having to isolate foreign proteins from leaf extracts. Several attempts have also been made to make plants transgenic for production of foreign proteins to be used as vaccine candidates. Mason *et al* (1996) have made transgenic tobacco and potato plants to successfully produce Norwalk virus capsid protein, which shows immunogenicity in mice. Wigdorowitz *et al.* (1999) have made transgenic alfalfa plants expressing an antigenic protein against FMDV and shown that animals immunised with purified antigen show immunogenicity against the virus.

There have been a few attempts at producing candidate vaccines against HIV-1 in plants. Yusibov *et al.* (1997) have used the coat protein of alfalfa mosaic virus as a carrier molecule to express antigenic peptides from HIV-1 (V3 loop). *In vitro* transcripts of recombinant virus with sequences encoding the antigenic peptides were synthesised from DNA constructs and used to inoculate tobacco plants. Recombinant virus particles were produced and purified and used for immunisation of mice. The antigens elicited specific virus-neutralizing antibodies in immunised mice. Zhang *et al.* (2000) used a tomato bushy stunt virus (TBSV) as an expression vector of HIV-1 p24 protein. This gene was introduced into the TBSV genome as an in-frame fusion with a 5' terminal portion of the TBSV coat protein ORF. Introduction into plants led to the accumulation of p24 fusion proteins in inoculated leaves.

SUMMARY OF THE INVENTION

According to a first embodiment of the invention, there is provided a method of producing an HIV-1 subtype C immunogenic protein or a related amino acid sequence, the method comprising the steps of:

introducing into a plant a tobacco mosaic virus vector or vector system including a nucleic acid sequence encoding the HIV-1 subtype C immunogenic protein or related amino acid sequence derived by substitution, deletion and/or insertion of one or more nucleotides, and/or extension or truncation of one or both ends thereof;

causing expression of the nucleic acid sequence in the plant; and

causing expression of the nucleic acid sequence in the plant; and

recovering the resulting HIV-1 subtype C immunogenic protein or related amino acid sequence produced within the plant.

The nucleic acid sequence preferably hybridises, under standard conditions, to at least a part of an isolated nucleic acid sequence coding for the immunogenic protein, or the complement of the nucleic acid sequence coding for an immunogenic protein or to its degenerate equivalents.

The immunogenic protein may be HIV-1 subtype C Pr55 Gag. In particular, the protein may be assembled into the form of virus-like particles (VLPs).

The nucleic acid sequence may be selected so as to cause or aid assembly of the immunogenic protein into virus-like particles.

The nucleic acid sequence may be at least part of the *gag* gene obtained from HIV-1 isolate DU422, assigned provisional accession no. 01032114 by the European Collection of Cell Cultures.

The plant may be a tobacco plant.

According to a second embodiment of the invention, there are provided HIV-1 subtype C immunogenic proteins or related amino acid sequences obtained from the method substantially as described above. The protein may be HIV-1 subtype C Pr55 Gag, and may be assembled into the form of virus-like particles (VLPs).

According to a further embodiment of the invention, there is provided a vector or vector system which expresses HIV-1 subtype C Pr55 Gag in tobacco plants.

The vector may be a tobacco mosaic virus vector.

According to yet a further embodiment of the invention, there is provided a recombinant vector system including a tobacco mosaic virus vector containing a nucleic acid sequence encoding an HIV-1 subtype C immunogenic protein or a related amino acid sequence derived by substitution, deletion and/or insertion of one or more nucleotides, and/or extension or truncation of one or both ends thereof; said nucleic acid

sequence hybridising, under standard conditions, to at least a part of an isolated nucleic acid sequence coding for the immunogenic protein, or the complement of the nucleic acid sequence coding for an immunogenic protein or to its degenerate equivalents.

The immunogenic protein may be HIV-1 subtype C Pr55 Gag.

The nucleic acid sequence may be selected to cause or aid assembly of the immunogenic protein into virus-like particles.

According to yet a further embodiment of the invention, there is provided a host cell including the vector or vector system substantially as described above.

The host cell is preferably a plant cell, and may be a tobacco plant cell.

According to yet a further embodiment of the invention, there is provided a vaccine for use in the treatment or prophylaxis of HIV infection in a mammal, the vaccine including virus-like particles produced substantially according to the method described above.

Preferably, the vaccine will be sufficient to induce an immunogenic response to the virus-like particles in a suitable susceptible host.

The vaccine may include a pharmaceutical excipient and/or adjuvant, and a therapeutically effective amount of the virus-like particles.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of the *gag* open reading frame of the HIV-1 genome;

Figure 2 shows a schematic representation of the matrix (MA) protein (p24) domain of the *gag* gene of Figure 1;

Figure 3 shows a schematic representation of the capsid (CA) protein (p24) domain of the *gag* gene of Figure 1;

Figure 4 shows a schematic representation of the nucleocapsid (NC) protein (p7) domain of the *gag* gene of Figure 1;

Figure 5 shows a schematic representation of the domain of the p6 protein domain of the *gag* gene of Figure 1;

Figure 6 shows the DNA sequence of the DU422 gag sequence used for cloning into pBSG1057. The bold underlining represents the *gag* gene and the dotted underlining represents the partial *pol* fragment;

Figure 7 shows a plasmid map of pBSG1057;

Figure 8 shows a plasmid map of pBSGgag6; and

Figure 9 shows a plasmid map of pBSGgagopt11.

DETAILED DESCRIPTION OF AN EMBODIMENT OF THE INVENTION

The invention will now be described in more detail with reference to a particular embodiment of the invention.

Cloning of *Gag* into TMV vector pBSG1057

The *Gag* gene was obtained from HIV-1 isolate DU422 (obtained from a South African sex worker cohort, and assigned provisional accession no. 01032114 by the European Collection of Cell Cultures) (Figure 6). It comprises of the entire *gag* gene and the first 57 bases of the *pol* gene. It was cloned into the *Eco*RI and *Sal*I restriction enzyme sites of an *E. coli* vector pGEM-T easy™. The ends of the gene were modified by PCR amplification such that *Pac*I and *Xho*I restriction enzyme sites were

attached to the 5' and 3' ends respectively, to facilitate cloning into the TMV vector pBSG1057 (Large Scale Biology Corporation) (Figure 7). Amplification products were re-cloned into pGEM-T easy™ and sequenced to verify the integrity of the restriction enzyme sites and the *gag* sequence.

The green fluorescent protein (GFP) gene sequence was excised from pBSG1057 by restriction enzyme digestion with *Pac*I and *Xho*I, and *gag* cloned into the TMV vector at these 2 sites to produce the clone pBSGgag6 (Figure 8).

Codon-optimisation of *gag* gene for *Nicotiana*

An additional strategy was undertaken to codon-optimise the *gag* gene for *Nicotiana*, synthesise it, and clone it using a similar strategy to that described above into the pBSG1057 TMV vector with the hope that this gene would enhance Gag protein expression when introduced into *N. benthamiana*. The codon-optimised gag gene was cloned into the *Pac*I and *Xho*I restriction enzyme sites of pBSG1057 after removal of GFP.
The resultant clone was called pBSGgagopt11 (Figure 9).

Transcription of pBSGgag6, pBSGgagopt11 and pBSG1057

mRNA of pBSGgag6, pBSGgagopt11 and pBSG1057 was produced using a Ribomax Transcription/translation kit (Promega). Ten micrograms of each plasmid was used per reaction.

Infection of *Nicotiana benthamiana* with recombinant TMV mRNA

The mRNA (50µl) was rubbed over an expanding leaf of 6-week old *N. benthamiana* plants using cotton-wool buds. Plants inoculated with the control pBSG1057 mRNA were monitored daily with a UV light for the appearance of GFP in both inoculated and upper leaves of the plants. Systemic spread of GFP was used as an indicator of systemic spread of recombinant TMV and leaves were sampled for detection of Gag protein by western blotting, EM analysis and ELISA.

Detection of Gag protein

Crude protein preparations were made by crushing up leaves in a mortar and pestle, filtration of remaining solid matter through cheesecloth, and addition of loading buffer.

Western blotting

The samples were boiled for 5 minutes and run on 10% SDS polyacrylamide gels to separate the proteins. Resolved proteins were transblotted from the SDS polyacrylamide gels onto nitrocellulose membranes. Membranes were probed with an anti-mouse p17 monoclonal antibody (Chemicon) at a dilution of 1:200.

EM analysis for Gag VLPs

Crude leaf extracts were centrifuged at low speed to pellet remaining unground tissue. Small amounts of leaf extract were dried on copper grids and immunotrapped with an anti-mouse p17 monoclonal antibody (Chemicon) at a dilution of 1:200. The grids were counterstained with uranyl acetate and looked at using a transmission electron microscope.

ELISA

Crude leaf extracts were centrifuged at low speed to pellet remaining unground tissue. Small amounts were diluted in 1 X PBS and used for detection of p24 antigen by using the Vironostika® HIV-1 antigen ELISA kit.

RESULTS

Baculovirus expression of HIV-1 subtype C gag VLPs

HIV-1 subtype C Gag VLPs were produced using the Bac-to-Bac® baculovirus expression system (Life Technologies). These provide a

relevant positive control for further protein (Gag) detection experiments and can be used to generate antibodies specific to HIV-1 subtype C Gag.

Because the cloned *gag* gene contains an N-terminal glycine residue sequence, it is expected that the recombinant protein be targeted to the host plasma membrane. VLPs thus formed were subsequently budded from the cell surface into the insect cell medium. VLPs were purified from the insect cell medium following a sucrose density gradient protocol for baculovirus-derived HIV VLPs detailed by Nermut *et al* (1994). The resulting protein band after centrifugation on the density gradient was extracted, dialysed in phosphate-buffered saline (PBS pH 7.4) overnight and small amounts used for SDS Page analysis, western blotting and EM analysis. VLPs of approximately 110 to 120 nm in diameter were visualized under the electron microscope, verifying successful gag VLP production. A single 55kD protein band was visualized in samples resolved on an SDS page gel, and a monoclonal and a polycolonal antibody to gag P17 protein were found to react positively using western blot analysis. Two additional monoclonal antibodies to gag P24 protein were tested subsequently against baculovirus-derived VLPs and reacted positively using western blot analysis.

Assaying for expression of *gag* in *N. benthamiana*

N. benthamiana plants were inoculated with mRNA transcripts of the pBSGgag6 and pBSGgagopt11 clones as well as with the TMV vector containing GFP (pBSG1057) as described previously. Water-inoculated plants served as negative controls. Plants were grown under normal growth conditions in plant rooms and monitored most days for the appearance of green fluorescent spots in the control plants inoculated with pBSG1057 mRNA transcripts, as well as for TMV symptoms in pBSGgag6-, pBSGgagopt11- and pBSG1057-inoculated plants. Leaves were periodically sampled for EM analysis as well as western blot analysis and ELISA on crude extracts.

TMV symptoms and GFP fluorescence:

Green fluorescent spots were visible under the UV light on the inoculated leaves of those infected with pBSG1057 mRNA transcript at 4 days post inoculation (dpi). Spread of the GFP spots to upper leaves was visible at 10 dpi. TMV symptoms were visible in the newer growth of pBSG1057-inoculated plants at 17 dpi and in the pBSGgag6- and pBSGgagopt11-inoculated plants at 24 dpi.

Western blotting

Lanes containing crude protein preparations from pBSGgag6- and pBSGgagopt11-inoculated leaves did not yield any positive result compared with baculovirus-produced gag which highlighted the presence of a 55kD protein.

EM analysis (immunotrapping):

EM analysis was performed using crude apical leaf extracts (35 dpi) ground up in PBS (pH 7.4) and trapped with a concentrated solution of p17 monoclonal antibodies. There was very little plant material present in the pBSG1057-inoculated sample (no particles ranging from 100 to 120 nm in size) and very few TMV particles. In the pBSGgag6-inoculated sample there were a large number of 100 to 110 nm-sized VLPs as well as quite a few TMV subunit-sized particles (25 nm). In the pBSGgagopt11-inoculated sample, there were similar shaped but less 100 to 110 nm-sized particles to those seen in the pBSGgag6-inoculated sample.

ELISA

Preliminary results of ELISA performed on crude preparations of protein showed the presence of gag protein in pBSGgagopt11-inoculated leaves at a level of 460pg/ml crude preparation protein solution.

Although the invention has been described above with reference to one particular embodiment, it is not intended that this should limit the invention to what has been described above.

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DATED THIS 4TH DAY OF NOVEMBER 2002

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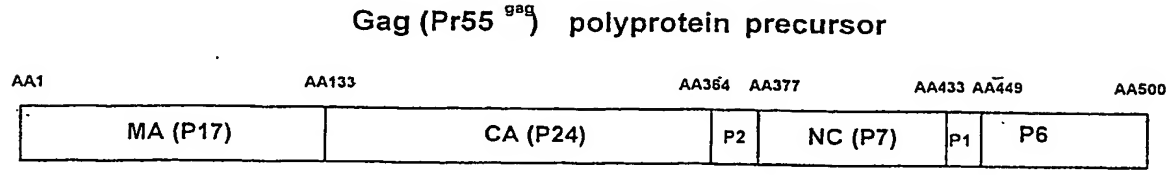

Figure 1

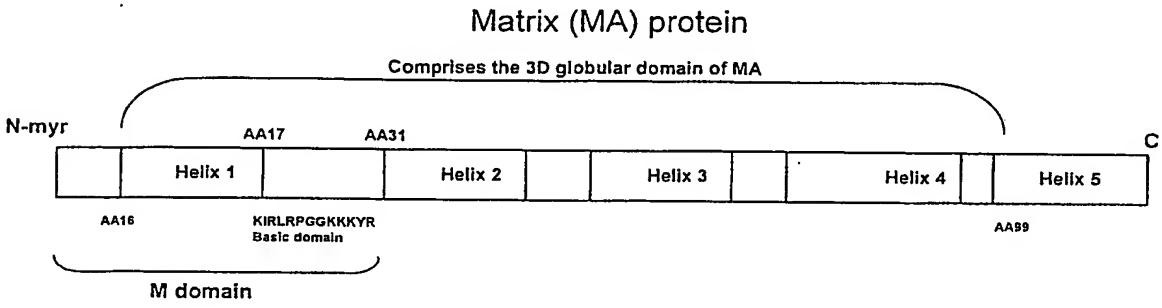

Figure 2

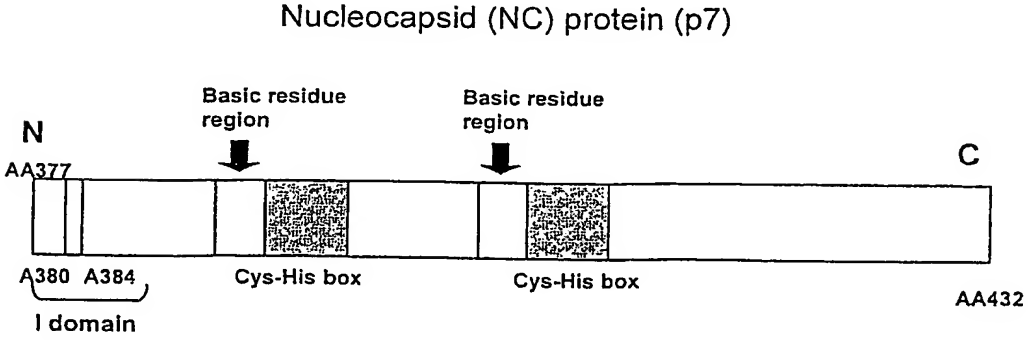

Figure 3

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Capsid (CA) protein (p24)

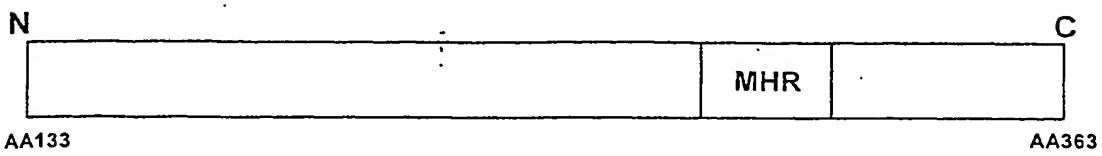

Figure 4

P6 protein

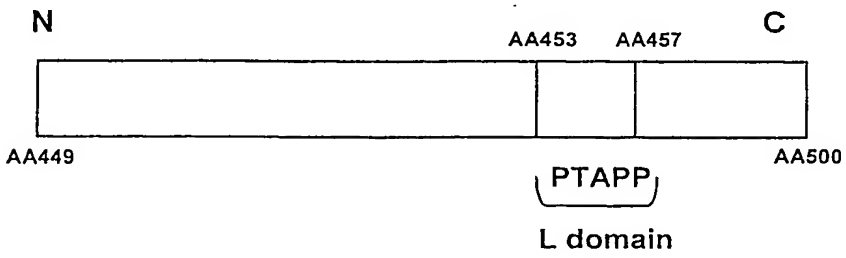

Figure 5

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181   CAAATAATGA AACAGCTACA ACCAGCTCTC CAGACAGGAA CAGAGGAACT TAAATCATTA
241   TACAACACAG TAGCAACTCT CTATTGTGTA CATGAAAAGA TAGAAGTACG AGACACCAAG
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661   CCTATTGCAC CAGGCCAGAT GAGAGAACCA AGGGGAAGTG ACATAGCAGG AACTACTAGT
721   ACCCTTCAGG AACAAATAGC ATGGATGACA AGTAACCCAC CTATTCCAGT GGGAGACATC
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1441  ACTTCCCTCA AATCACTCTT TGGCAGCGAC CCCTTGTCTC AATAAAAGTA GGGGGCCAGA
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Figure 6

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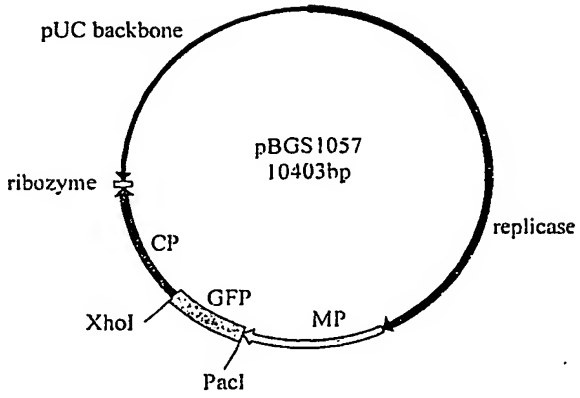

Figure 7

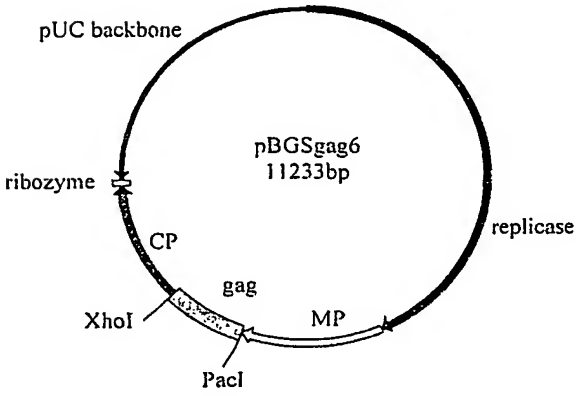

Figure 8

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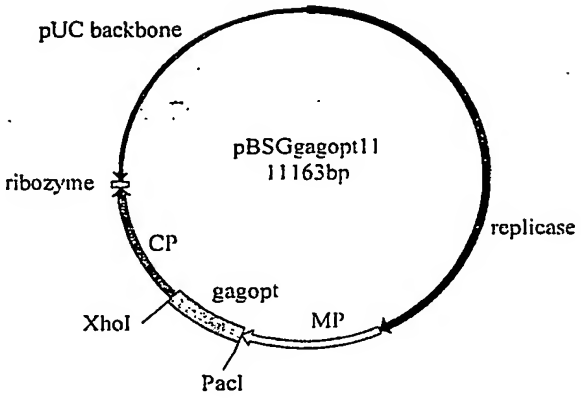

Figure 9

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